JIPB Journal of Integrative Plant Biology

A putative 6 trans-membrane nitrate transporter *OsNRT1.1b* plays a key role in rice under low nitrogen

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Abstract *OsNRT1.1a* is a low-affinity nitrate (NO_3^-) transporter gene. In this study, another mRNA splicing product, *OsNRT1.1b*, putatively encoding a protein with six transmembrane domains, was identified based on the rice genomic database and bioinformatics analysis. *OsNRT1.1a*/*OsNRT1.1b* expression in *Xenopus* oocytes showed *OsNRT1.1a*-expressing oocytes accumulated ^{15}N levels to about half as compared to *OsNRT1.1*b-expressing oocytes. The electrophysiological recording of *OsNRT1.1b*-expressing oocytes treated with 0.25 mM NO_3^- confirmed ^{15}N accumulation data. More functional assays were performed to examine the function of *OsNRT1.1b* in rice. The expression of both *OsNRT1.1a* and *OsNRT1.1b* was abundant in roots and downregulated by nitrogen (N) deficiency. The shoot biomass of transgenic rice plants with *OsNRT1.1a* or *OsNRT1.1b* overexpression increased under various N supplies under hydroponic conditions compared to wild-type (WT). The *OsNRT1.1a* overexpression lines showed increased plant N accumulation compared to the WT in 1.25 mM NH_4NO_3 and 2.5 mM NO_3^- or NH_4^+ treatments, but not in 0.125 mM NH_4NO_3. However, *OsNRT1.1b* overexpression lines increased total N accumulation in all N treatments, including 0.125 mM NH_4NO_3, suggesting that under low N condition, *OsNRT1.1b* would accumulate more N in plants and improve rice growth, but also that *OsNRT1.1a* had no such function in rice plants.

Keywords: Nitrate; nitrogen accumulation; *OsNRT1.1a/b*; overexpression; rice; 6 transmembrane transport protein
Citation: Fan X, Feng H, Tan Y, Xu Y, Miao Q, Xu G (2015) A putative 6 trans-membrane nitrate transporter *OsNRT1.1b* plays a key role in rice under low nitrogen. **J Integr Plant Biol** XX:XX–XX doi: 10.1111/jipb.12382
Edited by: Toru Fujiwara, University of Tokyo, Japan
Received May 26, 2015; **Accepted** Jul. 21, 2015
Available online on Jul. 29, 2015 at www.wileyonlinelibrary.com/journal/jipb

Research Article

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INTRODUCTION

Nitrate (NO_3^-) is very important as a source of nitrogen (N) for plants. Plant root cells absorb NO_3^- via various transporters from the soil and then reduce it to ammonium (NH_4^+), and assimilate NH_4^+ into organic N via the GOGAT cycle (Forde 2000). Plant NO_3^- uptake requires efficient transport systems at both low and high NO_3^- concentrations, and numerous studies have demonstrated that NO_3^- influx involves high- and low-affinity NO_3^- transport processes (Miller et al. 2007; Xu et al. 2012). Many families of membrane proteins are involved in NO_3^- uptake, allocation, and storage in plants such as the NO_3^-/peptide transporter family (NPF), NO_3^- transporter 2 family (NRT2), chloride channel family (CLC), and slow anion channel-associated homologs (SLAC/SLAH) (Krapp et al. 2014; Leran et al. 2014; Xia et al. 2014). The NPF transporters include the low-affinity NO_3^- transporter family (NRT1) and NO_3^-/peptide transporter family (Leran et al. 2014; Xia et al. 2014).

NRT1 transporters in higher plants contain 12 putative transmembrane (TM) regions with a large hydrophilic loop between TM6 and TM7 whose position is unique in higher plant NRT1 and rat PHTS (Chiang et al. 2004). In most animal NRT1 transporters, the long loop is located between TM9 and TM10, while it is between TM7 and TM8 in fungi. However, the function of the long hydrophilic loop of NRT1 transporters remains unclear. *AtNRT1.1* (or *CHL1*), as the first identified *NRT1* gene in plants, was isolated from *Arabidopsis* in 1978 (Doddema et al. 1978) and further confirmed using a transferred DNA-tagged *Arabidopsis* mutant in 1993 (Tsay et al. 1993). Using the *Xenopus* oocyte expression system, Tsay et al. (1993) showed that AtNRT1.1 (CHL1) is a proton-coupled NO_3^- transporter (Tsay et al. 1993). In addition, AtNRT1.1 (CHL1) exhibits two phases of NO_3^- uptake, with a K_m of 50 μM for the high-affinity phase and a K_m of 4 mM for the low-affinity phase, indicating that CHL1 is a dual-affinity NO_3^- transporter (Liu et al. 1999); the reported NRT1 transporter had a K_m above 4 mM NO_3^-. Recently, structural studies showed that AtNRT1.1 functions in the membrane as a dimer (Sun et al. 2014). Parker and Newstead (2014) used a 6 TM model to study the structure of AtNRT1.1 and demonstrated that the NO_3^-–proton symport model was a symmetry interaction of inward and outward open between the two 6 TM proteins in the AtNRT1.1 protein.

In rice, *OsNRT1.1* was identified as a low-affinity NO_3^- transporter gene with an affinity for NO_3^- of 9 mM ($K_m = 9$

mM) (Lin et al. 2000). By searching the cDNA database, *OsNRT1.1b*, putatively encoding a protein with only six TM domains, was considered a putative mRNA splicing product of *OsNRT1.1*. To distinguish between *OsNRT1.1b* and *OsNRT1.1* (Lin et al. 2000), we renamed *OsNRT1.1* as *OsNRT1.1a*. Based on AtNRT1.1 structural data (Parker and Newstead 2014; Sun et al. 2014), we hypothesized that one 6 TM NO_3^- transporter could function in plants through symmetry interactions of inward and outward open between protein dimers. To support these hypotheses, we tested the NO_3^- uptake function of *OsNRT1.1b* in *Xenopus* oocytes and rice plants.

RESULTS

Sequence analysis and expression pattern of *OsNRT1.1b*

OsNRT1.1 was identified as a low-affinity NO_3^- transporter gene that is constitutively expressed in roots (Lin et al. 2000). Based on the rice genomic database and bioinformatics analysis, we searched *OsNRT1.1* mRNA (AF140606) on the NCBI Web site and identified another mRNA splicing product, AK066920, which was further named *OsNRT1.1b*. To distinguish between *OsNRT1.1b* and *OsNRT1.1* (Lin et al. 2000), we renamed *OsNRT1.1* as *OsNRT1.1a*. OsNRT1.1a encoded a protein of 584 amino acids and 12 predicted TM domains, and a large hydrophilic loop existed between TM6 and TM7 (Figures 2A, S1). OsNRT1.1b consisted of 291 amino acids and six predicted TM domains, which shared the same amino acid sequences from 1 to 226 with OsNRT1.1a (Figures 2A, S1).

OsNRT1.1a is a root-specific gene with little or no expression in the shoot that remains relatively stable before and after NO_3^- induction (Lin et al. 2000). Furthermore, we detected *OsNRT1.1b* expression with different N supplies using reverse transcription–polymerase chain reaction (RT-PCR). Rice seedlings were hydroponically cultured in IRRI nutrient solution for 14 d, and then transferred to nutrient solutions containing different forms of nitrogen regimes, including standard N supply in IRRI solution 1.25 mM NH_4NO_3 (1.25 AN), N starvation (−N), 0.2 mM NO_3^- (0.2 N), 5 mM NO_3^- (5 N), 0.2 mM NH_4^+ (0.2 A) and 5 mM NH_4^+ (5 A) for another 14 d. Similar to *OsNRT1.1a*, *OsNRT1.1b* was expressed much more abundantly in roots than in aerial parts (Figure 2B, C). N starvation could decrease both *OsNRT1.1a* and *OsNRT1.1b* expression in roots (Figure 2B, C). Furthermore the real-time PCR results confirmed the semi-quantity RT–PCR data that the root mRNA level of *OsNRT1.1b* was higher in 0.2 N and 1.25 AN than other forms of N regimes treatment; however, *OsNRT1.1a* did not show significant difference among 1.25 AN, 0.2 N,5 N, 0.2 A, and 5 A conditions (Figure 2B, C).

OsNRT1.1b mRNA-injected oocytes showed NO_3^- uptake activity

To determine whether *OsNRT1.1b* encodes an NO_3^- transporter, *OsNRT1.1b* mRNA was injected into *Xenopus* oocytes. Two days after injection, the oocytes were used to record the membrane potential. Water-injected oocytes showed no response to NO_3^- (Figure 3A). However, when treated with 0.25 mM NO_3^-, oocytes injected with mRNA encoding *OsNRT1.1b* displayed NO_3^--induced changes in membrane potential from −33 to −20 mV, representing a depolarization of 13 mV (Figure 3B). The membrane potential could be restored when NO_3^- was removed (Figure 3B).

To examine the NO_3^- uptake activity of *OsNRT1.1b*, *OsNRT1.1b*-injected oocytes were incubated in 0.25 mM $^{15}NO_3^-$ (pH 7.4) 8 h for further NO_3^- uptake assays. Compared with water-injected oocytes, 100% of *OsNRT1.1b*-injected oocytes and 100% of *OsNRT1.1a*-injected oocytes exhibited ^{15}N accumulation (Figure 3C). Data spread analysis showed that almost 100% of the OsNRT1.1b RNA injected oocytes showed higher ^{15}N than OsNRT1.1a RNA injected oocytes (Figure 3C). As shown in Figure 3D, both *OsNRT1.1a*- and *OsNRT1.1b*-injected oocytes could absorb ^{15}N–NO_3^-, but *OsNRT1.1a*-expressing oocytes accumulated about half the ^{15}N as compared with *OsNRT1.1b*-expressing oocytes.

Generation of the transgenic rice overexpressing *OsNRT1.1a* and *OsNRT1.1b*

OsNRT1.1a and *OsNRT1.1b* overexpression lines were constructed by transforming pUbi–*OsNRT1.1a* and pUbi–*OsNRT1.1b* constructs into rice. More than 15 lines of the T_0 generation for each gene were obtained. Three independent transgenic lines of the T_2 generation for *OsNRT1.1a* (OEa1, OEa2, and OEa3) and *OsNRT1.1b* (OEb1, OEb2, and OEb3) were selected based on Southern blotting and RT–PCR analysis (Figure 4). RT–PCR analysis showed that in OEa1, OEa2, and OEa3 lines, the expression of *OsNRT1.1a* increased, while *OsNRT1.1b* did not change compared with the wild type (WT) (Figures 4A, S2). In OEb1, OEb2, and OEb3 lines, *OsNRT1.1b* transcript levels increased, but the *OsNRT1.1a* mRNA level did not change compared with the WT (Figures 4A, S2). OEa1 and OEb1 transgenic lines had two copies, while OEa2, OEa3, OEb2, and OEb3 had a single copy based on Southern blotting analysis (Figure 4B).

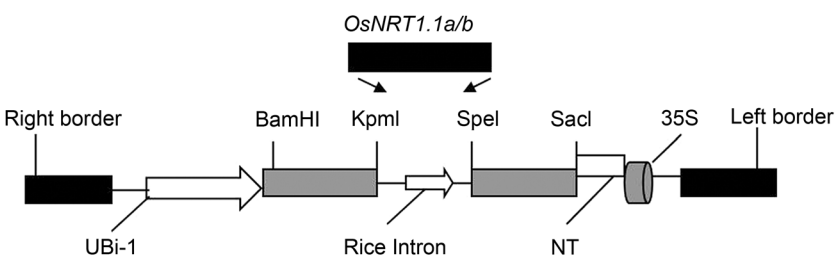

Figure 1. Schematic diagram of the pUbi–*OsNRT1.1a/b* expression vector for rice transformation
OsNRT1.1a/b cDNA was inserted in place of the rice intron in pTCK303 with *Kpn*I and *Spe*I sites.

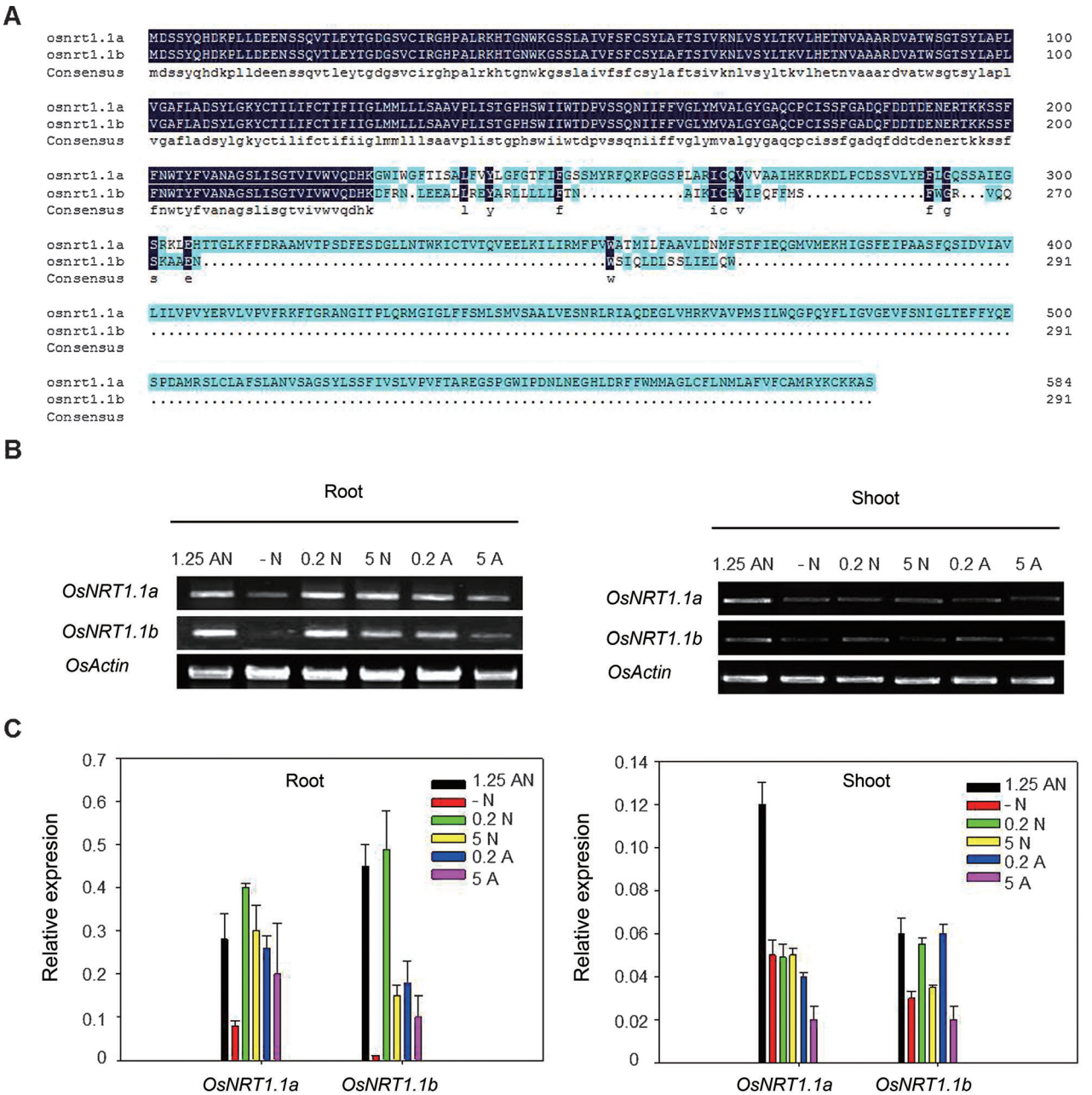

Figure 2. Amino acid sequence alignment and gene expression in *OsNRT1.1a* and *OsNRT1.1b*

(A) Alignment of OsNRT1.1a and OsNRT1.1b amino acid sequences. The picture was constructed using DNAMAN 5.2.2. **(B, C)** Expression of *OsNRT1.1a/b* in response to different nitrogen (N) regimes by semiquantitative RT-PCR (**B**) and real-time PCR (**C**). Rice seedlings were cultured in IRRI nutrient solution for 14 d and then transferred to nutrient solutions containing 1.25 mM NH_4NO_3 (1.25 AN), no N (–N), 0.2 mM NO_3^- (0.2 N), 5 mM NO_3^- (5 N), 0.2 mM NH_4^+ (0.2 A), and 5 mM NH_4^+ (5 A) for another 14 d. Total RNA was extracted from roots and shoots. *OsActin* was used as an internal control.

Overexpression of *OsNRT1.1b* significantly enhanced rice growth

Rice seeds of the WT, *OsNRT1.1a*, and *OsNRT1.1b* T_2 transgenic lines were cultured with ½ IRRI nutrient solution for 14 d and then treated with 1.25 mM NH_4NO_3 (1.25 AN), 0.125 mM NH_4NO_3 (0.125 AN), 2.5 mM NH_4^+ (2.5 A), and 2.5 mM NO_3^- (2.5 N) for another 14 d. Compared with the WT, *OsNRT1.1a* overexpression (OEa) lines showed increased shoot dry weight under all N supplies (Figure 5A) and increased root dry weight at 1.25 AN (Figures 5B, S3). Under 1.25 AN supply, the dry weight of OEa lines increased by 75 to 125% in shoots and 80 to 133% in roots compared with the WT (Figure 5A, B).

In *OsNRT1.1b* T2-overexpressed (OEb) lines, compared with the WT, shoot biomass was higher under all N supplies and root biomass was much higher in 1.25 AN and 2.5 A solutions (Figures 5C, D, S3). Shoot and root weight of OEb lines

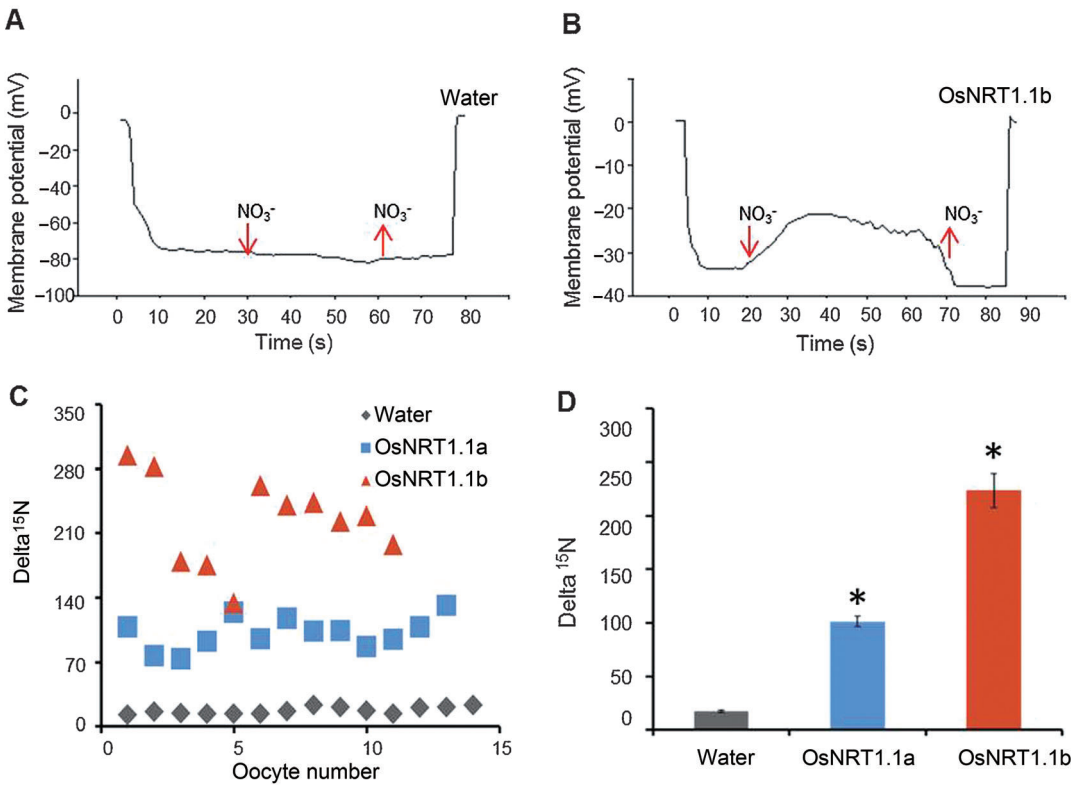

Figure 3. *OsNRT1.1a*- and *OsNRT1.1b*-injected *Xenopus* oocytes in response to nitrate (NO_3^-)
(A, B) NO_3^--induced changes in membrane potential for oocytes injected with water **(A)** or mRNA from *OsNRT1.1b* **(B)**. The recording was obtained from the oocytes injected with mRNA for 3 d. The injected oocytes were treated with 0.25 mM NO_3^- in pH 5.5 ND96 solution. **(C)** Data spread analysis of ^{15}N-nitrate influx for individual oocytes injected with water or RNA. Delta ^{15}N influx of individual oocytes injected with water (grey), RNA prepared for OsNRT1.1a (blue) and OsNRT1.1b (red) were compared. The data are from 12–14 cells. **(D)** $^{15}NO_3^-$ uptake in oocytes. A single oocyte was incubated in 0.25 mM $Na^{15}NO_3$ at pH 7.4 for 8 h, and then washed four times with cold 0.25 mM $NaNO_3$ before ^{15}N analysis. Data represent the average ± SE of six oocytes. $^*P \leq 0.05$ compared with the water-injected control. The example shown is representative of the results from two frogs.

increased respectively by 88 and 80% at 1.25 AN and 62 and 64% at 0.125 AN compared to the WT (Figure 5C, D). Both OEa and OEb lines showed no change in the root/shoot ratio under all N conditions (Figure 5E, F).

Overexpression of *OsNRT1.1a* enhanced N accumulation only at high N supplies, but *OsNRT1.1b*-overexpressing lines showed increased N accumulation at both high and low N supplies

To explore the cause of the enhanced biomass in *OsNRT1.1a* and *OsNRT1.1b* transgenic plants, we measured total N contents in WT and transgenic plants. Total N content in both shoots and roots showed no significant differences between OEa lines and the WT under different N treatments (Figure 6A, B). OEb lines showed a higher total N content in shoots and roots than the WT under 0.125 AN condition, except OEb1 root (Figure 6C, D). The pattern of nitrogen content in shoots and roots showed slightly different in OEb1 from OEb2 and OEb3 at this condition. Total plant N was calculated as the shoot N content multiplied by shoot biomass plus root N content multiplied by root biomass. Both OEa and OEb lines exhibited significantly greater accumulation in whole plants than the WT under all treatments, except OEa lines in 0.125 AN condition (Figure 6E, F). Under 0.125 AN condition, OEb lines accumulated more plant N compared with WT (Figure 6F); however, greater plant N accumulation of OEa was not observed in the same condition (Figure 6E). Under 0.125 AN supply, the N accumulation of OEb lines increased by 56 to 70% in plants compared with the WT and significantly more by 31 to 51% than OEa lines (Figure 6E, F).

The *OsNRT1.1a* overexpression lines showed no shift in the ratio of root N and shoot N from the WT at either 0.125 AN, 1.25 AN, 2.5 A, or 2.5 N supplies (Figure 6G). However, *OsNRT1.1b* overexpression lines decreased root N transfer to the shoot at 0.125 AN (Figure 6H), indicating that under low N condition, *OsNRT1.1b* accumulated more N in roots but that *OsNRT1.1a* had no such function in rice plants. While, as root/shoot biomass ratio of *OsNRT1.1b* overexpression did not change under 0.125 AN (Figure 5F), which means that the shoot growth was also increased as much as root even though the root/shoot N ratio was upregulated in *OsNRT1.1b* overexpression lines. The possible reason for this may be the N assimilation efficiency in the shoot of *OsNRT1.1b* overexpression lines was higher than that in their roots. However, it needs to be investigated further in future.

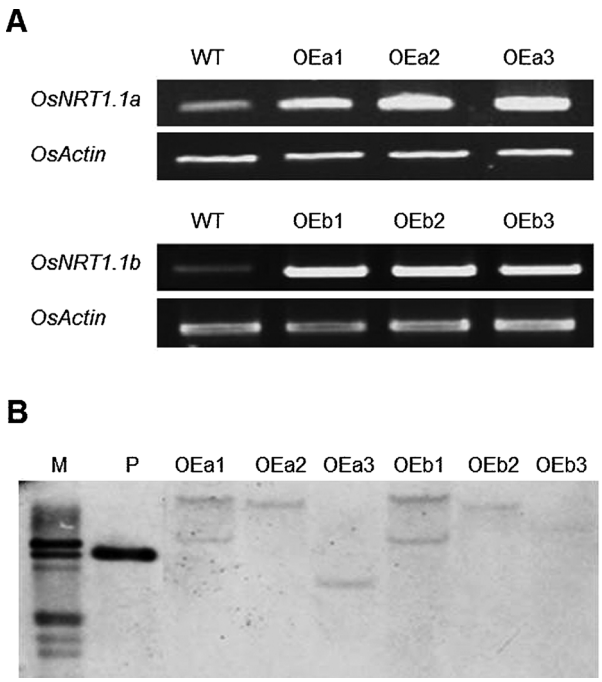

Figure 4. Molecular characterization of T_1 generation transgenic plants harboring the pUbi-*OsNRT1.1a/b* constructs
(A) The expression of *OsNRT1.1a* and *OsNRT1.1b* in wild-type (WT) and transgenic rice roots by RT-PCR. **(B)** Determination of copy numbers in transgenic rice plants by Southern blotting. OEa1, OEa2, and OE23 indicate *OsNRT1.1a* transgenic rice lines, and OEb1, OEb2, and OEb3 denote *OsNRT1.1b* transgenic rice lines. Southern blotting of *BamH*I and *Hind*III digests was performed using the WT as the negative control and the empty plasmid vector transgenic as the positive control.

DISCUSSION

OsNRT1.1b is a constitutively expressed NO_3^- transporter gene

Plant NRT1/PTR proteins could transport a wide variety of substrates and have recently been renamed as NPFs (Leran et al. 2014). In *Arabidopsis*, many NPFs showed NO_3^- transport activity (Chiu et al. 2004; Chopin et al. 2007; Segonzac et al. 2007; Almagro et al. 2008; Lin et al. 2008; Li et al. 2010). In rice, *OsNRT1.1* was the first NPF member in rice shown to function as a NO_3^- transporter after 3 h with a high NO_3^- treatment, but not a low NO_3^- treatment (Lin et al. 2000). Recently, *OsNPF2.4* (*OsNRT1.6*) belonging to cluster IV of the NPF family is a pH-dependent low-affinity NO_3^- transporter (Xia et al. 2014). In this study, *OsNRT1.1b* was found as another mRNA splicing product of *OsNRT1.1* (renamed *OsNRT1.1a*). OsNRT1.1a consisted of 584 amino acids and 12 TMs, while OsNRT1.1b comprised 291 amino acids and six TMs (Figures 2A, S1). *OsNRT1.1a* showed little or no NO_3^- transport activity in oocytes under low NO_3^- supply in previous reports (Lin et al. 2000), possibly because 3 h is not sufficient for accumulating ^{15}N or the current was too low to catch during the 2 V clamping (Lin et al. 2000). We extended the incubation time to 8 h for injected oocytes in 0.25 mM NO_3^-. Both *OsNRT1.1a*- and *OsNRT1.1b*-injected oocytes showed NO_3^- transport activity after an 8-h incubation under low NO_3^- supply, but *OsNRT1.1b*-injected oocytes could accumulate more $^{15}NO_3^-$ than *OsNRT1.1a*-injected oocytes (Figure 3B, C). For OsNRT1.1b, loss of the 6 TM protein significantly increased NO_3^- transport activity. Another example of two splicing NO_3^- transporters is reported to have different NO_3^- transport activity in rice. OsNRT2.3a and OsNRT2.3b, which were two mRNA splicing products of *OsNRT2.3*, showed different NO_3^- transport activity (Feng et al. 2011; Yan et al. 2011). OsNRT2.3a required a partner protein OsNAR2.1 to transport NO_3^-, but OsNRT2.3b could transport NO_3^- by itself in the *Xenopus* oocyte system (Feng et al. 2011; Yan et al. 2011).

OsNRT1.1a is expressed abundantly in roots and was not induced by NO_3^- (Lin et al. 2000). In our study, *OsNRT1.1b* showed similar expression patterns as *OsNRT1.1a*, which was constitutively expressed in roots (Figure 2B), even though the expression of *OsNRT1.1a/b* was quite low under N starvation condition, but was strongly induced under 0.2 mM nitrate or ammonium low nitrogen conditions (Figure 2B, C). The NRT1 family comprises both the constitutive and NO_3^--inducible component of the low-affinity NO_3^- transport system in *Arabidopsis* (Tsay et al. 1993; Huang et al. 1996, 1999). *AtNRT1.1* (*CHL1*), the NO_3^--inducible component, is involved in both low- and high-affinity NO_3^- uptake (Wang et al. 1998; Liu et al. 1999). *AtNRT1.2*, the constitutive component, exhibits only low-affinity uptake activities (Huang et al. 1999; Liu et al. 1999), similar to *OsNRT1.1a* and *OsNRT1.1b* (Figure 2B).

OsNRT1.1b can increase plant N accumulation under low N conditions

Although OsNRT1.1a was identified as a NO_3^- transporter in *Xenopus* oocytes (Lin et al. 2000) and injected *OsNRT1.1b* mRNA could transport NO_3^- under low NO_3^- supply (Figure 3), its biological function in plants had not been investigated. In this study, T_2 overexpressed transgenic lines of *OsNRT1.1a* and *OsNRT1.1b* were obtained to test gene function in rice. Overexpression of *OsNRT1.1a* stimulated shoot growth under all N conditions and root growth only under 1.25 mM NH_4NO_3 conditions (Figure 5A, B). However, *OsNRT1.1b* overexpression enhanced shoot growth in all N treatments and root growth in both 1.25 mM NH_4NO_3 and 2.5 mM NH_4^+ treatments, also increased the total N content in shoots and root under 0.125 mM NH_4NO_3 conditions (Figures 5C, D, 6C, D). These results suggested that OEb absorbed more nitrogen from external solutions. However the constitutive overexpression of *OsNRT2.1* in rice only enhanced vegetative growth, but did not affect NO_3^- uptake (Hisato et al. 2009).

It was very surprising that different nitrogen form treatments had significantly different effects on root growth for OEa and OEb lines, but not on shoots. In detail, we observed that OEa lines could show super root growth only under 1.25 AN, the mixture of nitrate and ammonium condition. None of the pure nitrate or ammonium or low nitrogen treatments could improve the OEa root growth, compared with WT. However, more interestingly we found the root growth of OEb lines could become greater not only under 1.25 AN but also under pure ammonium conditions. Whereas neither low nitrogen nor pure nitrate induced this super phenotype of OEb lines, compared with WT. Why pure nitrate failed to induce the root super growth of OEa and OEb

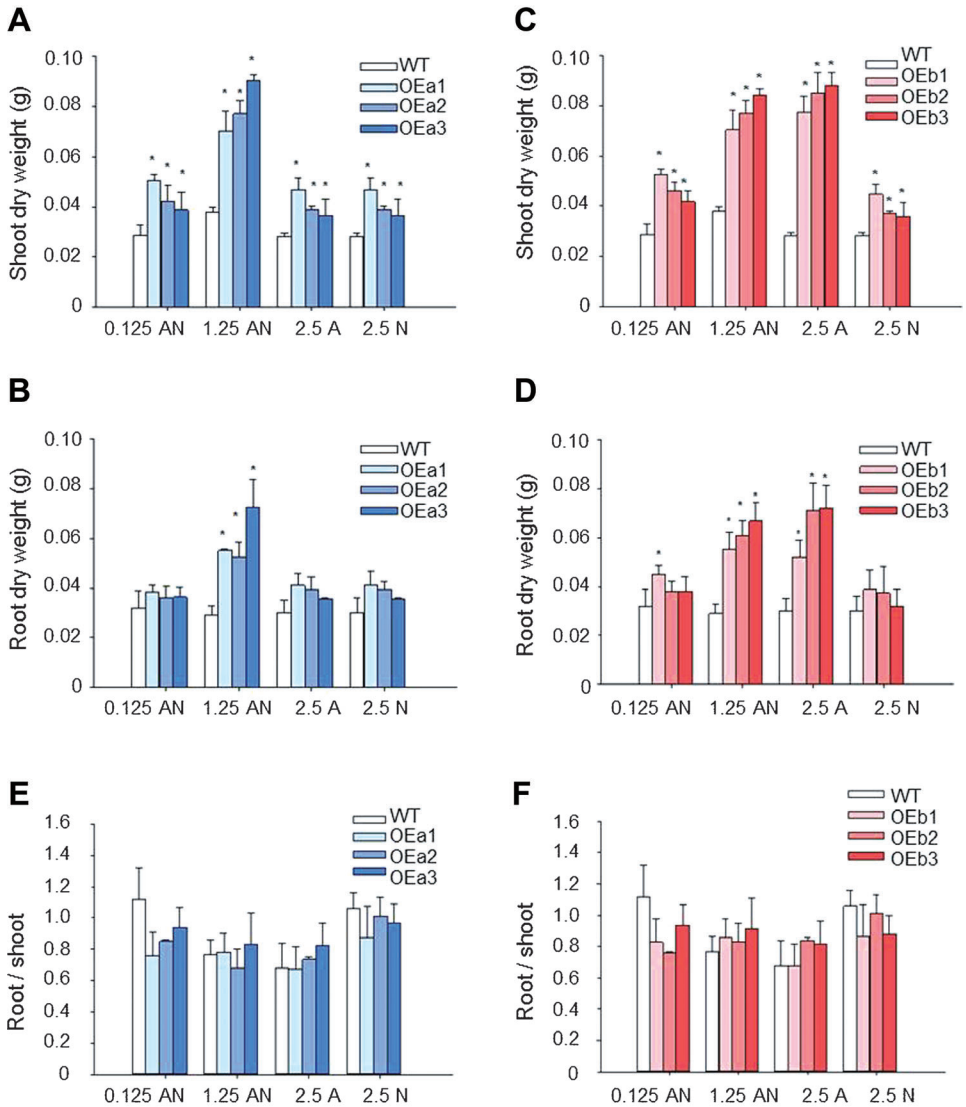

Figure 5. Growth of *OsNRT1.1a* and *OsNRT1.1b* transgenic lines compared with the wild type (WT) under different nitrogen supply conditions

(A) Shoot dry weight and **(B)** root dry weight in *OsNRT1.1a* transgenic lines. **(C)** Shoot dry weight and **(D)** root dry weight in *OsNRT1.1b* transgenic lines. Root/shoot in *OsNRT1.1a transgenic* lines **(E)** and *OsNRT1.1b* transgenic lines **(F)**. Root/shoot = root dry weight/shoot dry weight. The treatments were labeled as 0.125 mM NH_4NO_3 (0.125 AN), 1.25 mM NH_4NO_3 (1.25 AN), 2.5 mM NH_4^+ (2.5 A), and 2.5 mM NO_3^- (2.5 N). *Significant difference at the 0.05 probability level according to the LSD test ($n = 4$) estimated using a one-tailed ANOVA between WT and T_2 overexpression lines; data represent means ± SE.

lines is still unknown, even though it could be done at shoot growth. We guessed that the reason that low nitrogen treatment improved root growth of OEb lines rather than OEa lines was linked to the OsNRT1.1b function in oocytes, i.e. under low nitrate conditions, oocytes expressing OsNRT1.1b could respond to 0.25 mM nitrate quite well and absorbed more ^{15}N-nitrate than OsNRT1.1a under 0.25 mM nitrate supply. However, it was still hard to explain that the root growth of OEb could be improved under pure ammonium condition but that did not happen on OEa lines.

Furthermore, we calculated plant N accumulation and found that when supplied with high N, both *OsNRT1.1a* and *OsNRT1.1b* overexpression lines showed higher plant N accumulation than the WT (Figure 6E, F). However, under 0.125 AN conditions, overexpression of *OsNRT1.1b* showed increased plant N accumulation compared to the WT (Figure 6F). One possible mechanism was *OsNRT1.1b* could accumulate more ^{15}N than OsNRT1.1a under a low NO_3^- supply in oocytes, and therefore *OsNRT1.1b* may contribute to plant N accumulation under a low N supply. However, for high N conditions, there was no such difference between OEa and OEb lines.

AtNRT1.1 is a NO_3^- transporter present in the membrane as a dimer (Sun et al. 2014). The dimerization switch model was operated by the phosphorylation site at T101 of AtNRT1.1 (Sun et al. 2014). When AtNRT1.1 functioned as a high affinity

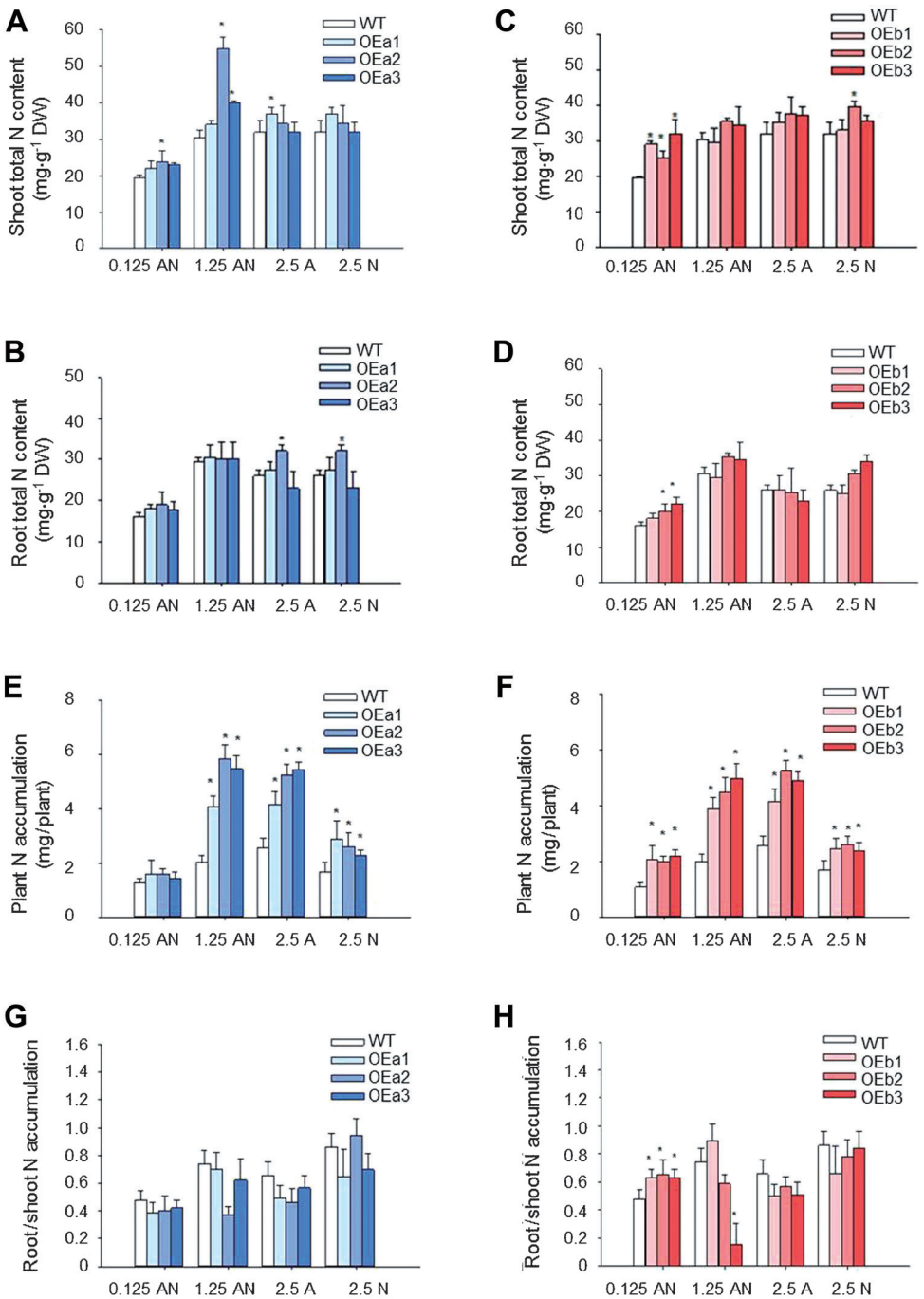

Figure 6. Total nitrogen (N) content and N accumulation of wild-type (WT) and transgenic lines with different N supplies
(A) Shoot N content and **(B)** root N content in *OsNRT1.1a* overexpression lines. **(C)** Shoot N content and **(D)** root N content in *OsNRT1.1b* overexpression lines. Plant N accumulation in *OsNRT1.1a* transgenic lines **(E)** and *OsNRT1.1b* transgenic lines **(F)**. Root N accumulation/shoot N accumulation in *OsNRT1.1a* transgenic lines **(G)** and *OsNRT1.1b* transgenic lines **(H)**. The treatments were labeled as 0.125 mM NH_4NO_3 (0.125 AN), 1.25 mM NH_4NO_3 (1.25 AN), 2.5 mM NH_4^+ (2.5 A), and 2.5 mM NO_3^- (2.5 N). *Significant difference at the 0.05 probability level according to the LSD test ($n = 4$) estimated using one-tailed ANOVA between WT and T_2 overexpression lines; data represent means ± SE.

transporter in plants, T101 site was phosphorylated and AtNRT1.1 dimer was decoupled. When T101 site of AtNRT1.1 was dephosphorylated the AtNRT1.1 protein was coupled into dimer and working as a low affinity transporter (Sun et al. 2014). We scanned the potential phosphorylation site in OsNRT1.1a and OsNRT1.1b by scansite software online (http://scansite.mit.edu/motifscan_seq.phtml). The prediction results showed that both OsNRT1.1a and OsNRT1.b had two potential

phosphorylation sites at S199 and T204 (Figure S5). That meant for both OsNRT1.1a and OsNRT1.1b they might be switched between dimer or mono protein in cells. Furthermore they might show a switch between low affinity and high affinity function. However, as Lin et al. (2000) presented that OsNRT1.1 had only one low affinity activity in oocytes no high affinity function, it defined the possibility of OsNRT1.1a could be switched between low and high affinity. But our oocytes data suggested this possibility may exist for OsNRT1.1b, since OsNRT1.1b could respond to low nitrate quite well (Figure 3B).

The NO_3^-–proton symport model of AtNRT1.1 was a symmetrical interaction of inward and outward open between the two 6 TM proteins in the AtNRT1.1 protein (Parker and Newstead 2014). Parker and Newstead (2014) used this 6 TM model to explain the mechanism for the effect of phosphorylation at T101 of this 6 TM on nitrate transport that was compared to the wild-type protein, the T101D of AtNRT1.1 increased nitrate uptake significantly. Therefore the phosphorylation site T101 controlled the functional activity of AtNRT1.1. Compared with the 12 TM topology of OsNRT1.1a, OsNRT1.1b contained only six TMs. However, our data suggested that OsNRT1.1b was expressed strongly under low nitrogen in rice (Figure 2B, C) and did function as an NO_3^- transporter during low nitrate supply (Figure 3). Overexpression of OsNRT1.1b could increase plant N accumulation in rice plants (Figures 5, 6). Based on structural studies of AtNRT1.1, we hypothesized that OsNRT1.b may perform a NO_3^- transporter function as a dimer in the cell, through the phosphorylation on S199 or T204. Furthermore this phosphorylation should increase the nitrate uptake based on the finding of Parker and Newstead 2014. This hypothesis fitted well to our nitrate uptake data as OsNRT1.1b had a much better nitrate uptake than OsNRT1.1a (Figure 3D). More protein analysis is needed in the future in order to understand better the different behaviors of OsNRT1.1a and OsNRT1.1b in plants.

MATERIALS AND METHODS

Plant growth conditions

Seed sterilization and the basal IRRI nutrient solution composition for seedling growth were described previously (Li et al. 2006). Two-week-old seedlings with uniform size and vigor were transferred into pots. For different N treatments, 0.125 or 1.25 mM NH_4NO_3, 1.25 mM $Ca(NO_3)_2$, and 1.25 mM $(NH_4)_2SO_4$ were added as N sources. Other nutrients were supplied as the IRRI nutrient solution with 0.3 mM KH_2PO_4, 0.35 mM K_2SO_4, 1 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM Na_2SiO_3, 20 μM NaFe–EDTA, 20 μM H_3BO_3, 9 μM $MnCl_2 \cdot 4H_2O$, 0.32 μM $CuSO_4 \cdot 5H_2O$, 0.77 μM $ZnSO_4 \cdot 7H_2O$, and 0.39 μM $Na_2MoO_4 \cdot 2H_2O$, pH 5.5. To inhibit nitrification, 7 μM dicyandiamide (DCD–$C_2H_4N_4$) was mixed into all solutions. All seedlings were grown in a greenhouse with a 16-h light (30 °C)/8-h dark (22 °C) photoperiod, and the relative humidity was approximately 70%. The nutrient solution was refreshed every 2 d.

Semiquantative RT-PCR and real-time PCR assay

Total RNA extraction from different rice tissues and RT-PCR using specific primers for *OsNRT1.1a* and *OsNRT1.1b* and the internal standard gene *OsActin* were performed as described previously (Feng et al. 2011). Amplification of real-time quantitative PCR products was performed with a single Color Real-Time PCR Detection System (MyiQ Optical Module; Bio-Rad) in a reaction mixture of 20 mL of SYBR Green master mix (SYBR PremixEx Tag TMII; TaKaRa Bio; http://www.takara-bio.com) according to the manufacturer's instructions (TaKaRa Biotechnology). The target genes and OsActin standards in 1:10, 1:100, and 1:1,000 dilutions were always present in the experiments (Tsuchiya et al. 2004). All primers used for semiquantitative RT-PCR and real-time PCR are listed in Table S1.

Functional analysis of *OsNRT1.1a* and *OsNRT1.1b* in *Xenopus laevis* oocytes

OsNRT1.1a and *OsNRT1.1b* cDNAs were subcloned into the oocyte expression vector pT7Ts and linearized using *Xba*I. mRNA was then synthesized *in vitro* using the mMESSAGE mMACHINE T7 Kit (Ambion, Austin, TX, USA). Oocytes were injected with 50 ng of mRNA, as described previously (Tong et al. 2000; Xia et al. 2014). Water-injected oocytes were used as controls. Oocytes were incubated in ND96 solution with antibiotics for 2 or 3 d (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES 50 μg ml^{-1} gentamycin and 100 μg ml^{-1} streptomycin, pH 7.4). Membrane potential recording was performed as described previously (Tong et al. 2000). Injected oocytes were incubated in 0.25 mM $Na^{15}NO_3$ ND96 solution for 8 h at pH 7.4, and then washed with cold 0.25 mM $NaNO_3$ ND96 solution quickly four times. A single oocyte was transferred to an empty tin capsule and then dried at 60 °C for 1 week to a constant weight. ^{15}N was measured using a continuous-flow isotope ratio mass spectrometer coupled to a C–N elemental analyzer (ANCA-GSL MS; PDZ Europa, Northwich, Cheshire, UK).

Generation and identification of *OsNRT1.1a* and *OsNRT1.1b* overexpression rice lines

The open reading frames of *OsNRT1.1a* and *OsNRT1.1b* were amplified by PCR and ligated into the ubiquitin promoter of the pTCK303 vector (Figure 1). For OsNRT1.1a, the forward primer was 5′- taatggatccCATTCTCTCGGACATTAACCT and the right primer was 5′-taatgagctcTTCCACCACCATTATATTGC. For OsNRT1.1b, the forward primer was 5′-taatggatccTTGGAGCTC-CACCGC and the right primer was 5′- taatgagctcCCCCCCCT CGAAGG.

The constructs were obtained and transformed into rice callus using *Agrobacterium tumefaciens* (strain EHA105), as described previously (Ai et al. 2009). More than 15 individual T_0 transgenic lines were obtained to verify the levels of *OsNRT1.1a* or *OsNRT1.1b* overexpression. Three independent lines of the T_1 generation were selected from both pUbi–*OsNRT1.1a* (OEa1, OEa2, and OEa3) and pUbi–*OsNRT1.1b* (OEb1, OEb2, and OEb3) transgenic lines, while one or two copies of the T-DNA insertion were used for further analyses. The copy numbers in the T_1 generation were performed by Southern blotting (DIG High Prime DNA Labelling and Detection Starter Kit I; Roche, http://www.roche.com/index.htm). The fragment of the coding sequences of the hygromycin genes labeled with digoxigenin was used as a probe, which was prepared by PCR according to the manufacturer's instructions (Roche).

Measurement of biomass, total N content, and accumulation

All plants (WT and T_2 transgenic lines) were harvested after different treatments and then dried at 105°C for 30 min, after which shoots and roots were dried at 75°C for 3 d. The dry weight was recorded as biomass. The root to shoot ratio was calculated as the root dry weight divided by the shoot dry weight. Total N content was measured as described previously (Cai et al. 2012). The plant accumulation N = (shoot N content * shoot biomass) + (root N content * root biomass). Four replicates were used for the calculation.

Statistical analysis

Data were analyzed by ANOVA using the SPSS 10 program (SPSS, Chicago, IL, USA). The asterisks on the histograms between the transgenic plants and WT and/or different treatments indicate their statistical difference at $P \leq 0.05$.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (31172013, 31372122 and 31401938).

AUTHOR CONTRIBUTIONS

X.F. and Q.M. performed most of the research. H.F. drafted the manuscript and part of oocyte expression experiment. Y.T. carried out gene expression experiments, Y.X. performed total N analyses. G.X. revised the manuscript. X.F. designed the experiment, supervised the study, and revised the manuscript.

REFERENCES

Ai P, Sun S, Zhao J, Fan X, Xin W, Guo Q, Yu L, Shen QR, Wu P, Miller AJ, Xu G (2009) Two rice phosphate transporters, OsPht1;2 and OsPht1;6, have different functions and kinetic properties in uptake and translocation. **Plant J** 57: 798–809

Almagro A, Lin SH, Tsay YF (2008) Characterization of the *Arabidopsis* nitrate transporter NRT1.6 reveals a role of nitrate in early embryo development. **Plant Cell** 20: 3289–3299

Cai J, Chen L, Qu H, Lian J, Liu W, Hu YB, Xu G (2012) Alteration of nutrient allocation and transporter genes expression in rice under N, P, K, and Mg deficiencies. **Acta Physiol Plant** 34: 939–946

Chiang CS, Stacey G, Tsay YF (2004) Mechanisms and functional properties of two peptide transporters, AtPTR2 and fPTR2. **J Biol Chem** 279: 30150–30157

Chiu CC, Lin CS, Hsia AP, Su RC, Lin HL, Tsay YF (2004) Mutation of a nitrate transporter, AtNRT1: 4, results in a reduced petiole nitrate content and altered leaf development. **Plant Cell Physiol** 45: 1139–1148

Chopin F, Orsel M, Dorbe MF, Chardon F, Truong HN, Miller AJ, Krapp A, Daniel-Vedele F (2007) The *Arabidopsis* ATNRT2.7 nitrate transporter controls nitrate content in seeds. **Plant Cell** 19: 1590–1602

Doddema H, Hofstra JJ, Feenstra WJ (1978) Uptake of nitrate by mutants of *Arabidopsis thaliana*, disturbed in uptake or reduction of nitrate I. Effect of nitrogen source during growth on uptake of nitrate and chlorate. **Physiol Plant** 43: 343–350

Feng H, Yan M, Fan X, Li B, Shen Q, Miller AJ, Xu G (2011) Spatial expression and regulation of rice high-affinity nitrate transporters by nitrogen and carbon status. **J Exp Bot** 62: 2319–2332

Forde BG (2000) Nitrate transporters in plants: Structure, function and regulation. **Biochim Biophys Acta** 1465: 219–235

Katayama H, Mori M, Kawamura Y, Tanaka T, Mori M, Hasegawa H (2009) Production and characterization of transgenic rice plants carrying a high-affinity nitrate transporter gene (*OsNRT2.1*). **Breeding Sci** 59: 237–243

Huang NC, Chiang CS, Crawford NM, Tsay YF (1996) CHL1 encodes a component of the low-affinity nitrate uptake system in *Arabidopsis* and shows cell type-specific expression in roots. **Plant Cell** 8: 2183–2191

Huang NC, Liu KH, Lo HJ, Tsay YF (1999) Cloning and functional characterization of an *Arabidopsis* nitrate transporter gene that encodes a constitutive component of low-affinity uptake. **Plant Cell** 11: 1381–1392

Jain M, Tyagi AK, Khurana JP (2006) Genome-wide analysis, evolutionary expansion, and expression of early auxin-responsive SAUR gene family in rice (*Oryza sativa*). **Genomics** 88: 360–371

Krapp A, David LC, Chardin C, Girin T, Marmagne A, Leprince AS, Chaillou S, Ferrario-Mery S, Meyer C, Daniel-Vedele F (2014) Nitrate transport and signaling in *Arabidopsis*. **J Exp Bot** 65: 789–798

Leran S, Varala K, Boyer JC, Chiurazzi M, Crawford N, Daniel-Vedele F, David L, Dickstein R, Fernandez E, Forde B, Gassmann W, Geiger D, Gojon A, Gong JM, Halkier BA, Harris JM, Hedrich R, Limami AM, Rentsch D, Seo M, Tsay YF, Zhang M, Coruzzi G, Lacombe B (2014) A unified nomenclature of NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER family members in plants. **Trends Plant Sci** 19: 5–9

Li BZ, Xin WJ, Sun SB, Shen QR, Xu GH (2006) Physiological and molecular responses of nitrogen-starved rice plants to re-supply of different nitrogen sources. **Plant Soil** 287: 145–159

Li JY, Fu YL, Pike SM, Bao J, Tian W, Zhang Y, Chen CZ, Zhang Y, Li HM, Huang J, Li LG, Schroeder JI, Gassmann W, Gong JM (2010) The *Arabidopsis* nitrate transporter NRT1.8 functions in nitrate removal from the xylem sap and mediates cadmium tolerance. **Plant Cell** 22: 1633–1646

Lin CM, Koh S, Stacey G, Yu SM, Lin TY, Tsay YF (2000) Cloning and functional characterization of a constitutively expressed nitrate transporter gene, OsNRT1, from rice. **Plant Physiol** 122: 379–388

Lin SH, Kuo HF, Canivenc G, Lin CS, Lepetit M, Hsu PK, Tillard P, Lin HL, Wang YY, Tsai CB, Gojon A, Tsay YF (2008) Mutation of the Arabidopsis NRT1.5 nitrate transporter causes defective root-to-shoot nitrate transport. **Plant Cell** 20: 2514–2528

Liu KH, Huang CY, Tsay YF (1999) CHL1 is a dual-affinity nitrate transporter of *Arabidopsis* involved in multiple phases of nitrate uptake. **Plant Cell** 11: 865–874

Miller AJ, Fan X, Orsel M, Smith SJ, Wells DM (2007) Nitrate transport and signalling. **J Exp Bot** 58: 2297–2306

Parker JL, Newstead S (2014) Molecular basis of nitrate uptake by the plant nitrate transporter NRT1.1. **Nature** 507: 68–72

Segonzac C, Boyer JC, Ipotesi E, Szponarski W, Tillard P, Touraine B, Sommerer N, Rossignol M, Gibrat R (2007) Nitrate efflux at the root plasma membrane: Identification of an *Arabidopsis* excretion transporter. **Plant Cell** 19: 3760–3777

Sun J, Bankston JR, Payandeh J, Hinds TR, Zagotta WN, Zheng N (2014) Crystal structure of the plant dual-affinity nitrate transporter NRT1.1. **Nature** 507: 73–77

Tong Y, Zhou J, Li Z, Miller AJ (2005) A two-component high-affinity nitrate uptake system in barley. **Plant J** 10: 1365–1374

Tsay YF, Schroeder JI, Feldmann KA, Crawford NM (1993) The herbicide sensitivity gene *CHL1* of *Arabidopsis* encodes a nitrate-inducible nitrate transporter. **Cell** 72: 705–713

Wang R, Liu D, Crawford NM (1998) The *Arabidopsis* CHL1 protein plays a major role in high-affinity nitrate uptake. **Proc Natl Acad Sci USA** 95: 15134–15139

Xia X, Fan X, Wei J, Feng H, Qu H, Xie D, Qu H, Xie D, Miller AJ, Xu G (2014) Rice nitrate transporter OsNPF2 4 functions in low-affinity acquisition and long-distance transport. **J Exp Bot** 66: 317–331

Xu G, Fan X, Miller AJ (2012) Plant nitrogen assimilation and use efficiency. **Annu Rev Plant Biol** 63: 153–182

Yan M, Fan X, Feng H, Miller AJ, Shen Q, Xu G (2011) Rice OsNAR2.1 interacts with OsNRT2.1, OsNRT2.2 and OsNRT2.3a nitrate transporters to provide uptake over high and low concentration ranges. **Plant Cell Environ** 34: 1360–1372

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. Transmembrane topology of OsNRT1.1a and OsNRT1.1b The figure was generated using the ConPred II program (http://bioinfo.si.hirosaki-u.ac.jp/~ConPred2/).

Figure S2. Expression of *OsNRT1.1b* in pUbi–*OsNRT1.1a* transgenic lines and *OsNRT1.1a* in pUbi–*OsNRT1.1b* transgenic lines compared to the wild type using RT-PCR in roots

Figure S3. Plant growth phenotype of *OsNRT1.1a* overexpression lines under different nitrogen supply conditions

Figure S4. Plant growth phenotype of *OsNRT1.1b* overexpression lines under different nitrogen supply conditions

Figure S5. Phosphorylation site scanning of OsNRT1.1a and OsNRT1.1 b

Table S1. *OsNRT1.1a* and *OsNRT1.1b* primers for RT-PCR